MBL Microbial Diversity Course 2009

Project Report

MICROBIAL THERMOSENSING AND THERMOTAXIS

Julia Rosa de Rezende

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Corresponding address:

Center for Geomicrobiology
Aarhus University
Julia.rezende@biology.au.dk

Abstract

Temperature is one of the most relevant environmental parameters, with dramatic impact on the physiology and ecology of any living being. It is expected, although scarcely studied in the microbial world, that most or all organisms are able to sense spatial temperature gradients or sudden rises and drops. In case of motile microorganisms, they may also readjust their location, moving to higher or lower temperatures, i.e. showing thermotaxis. This project attempts to establish an experimental setup for microbial thermotaxis assays, observe colony formation patterns in a temperature gradient and investigate thermotaxis ability on various strains of mesophilic and thermophilic bacteria isolated from seawater, brackish water, freshwater and soil environments in Woods Hole.

1. Introduction

Temperature is one of the most relevant parameters that influence the physiology of organisms. An ideal temperature helps to optimize rates of metabolism, growth and reproduction, improving the evolutionary fitness of a population. Physiological profiles must be readjusted following temperature variations, both in micro and macro scales. In many environments, it could be an advantage to be able to move according to the temperature changes, e.g. hydrothermal sediments, with steep temperature gradients in few centimeters or millimeters, or exposed soil and mats in tidal environments, with considerable temperature variation throughout day and night.

Therefore it is fairly expected that most – if not all – microorganisms are able to sense temperature and respond to changes in a certain level. This response could be recognized by modifications in intracellular processes or, for motile cells, also observed as changes in their location – i.e. thermotaxis. Such phenomenon has been widely studied in eukaryotes, such as *Dictyostelium discoideum* (Poff and Skokut 1977), *Caenorhabditis elegans* (Ramot *et al.* 2008), mammalian sperm cells (Bahat and Eisenbach 2006), but scarcely studied among Bacteria and Archaea.

Another intriguing question is whether the optimum temperature determined in laboratory by measuring growth rate is always the favorite temperature for an organism, if it has the chance to choose. Fast growth leads to fast depletion of nutrients and stressful metabolism, hence there could be conditions in which searching for lower temperatures could be an advantage. Additionally, various enzymes have different temperature optima, so escaping from the optimum temperature for growth could promote different metabolic patterns.

Up to now it has been reported that the thermotaxis mechanism in *E. coli* is closely related to that of chemotaxis, as cells search for an optimal combination of nutrients and temperature (Maeda *et al.* 1976, Salman and Libchaber 2007). Maeda *et al.* (1976) showed that *E. coli* cells in a microslide chamber subjected to a gradient between 20 and 39˚C first accumulated around 34˚C and then moved slowly towards lower temperatures. However, the authors state that the temperature gradient may have caused an oxygen gradient as a secondary effect, so the thermal response could have been influenced by a chemotactic response against oxygen.

Salman and Libchaber (2007) showed that *E. coli* populations change their temperature preference depending on their chemical environment and population density: cells grown below a critical population density (ca. $2x10^8$ cells cm^{-3}) tend to accumulate in higher temperatures than cells grown to density above critical, which tend to escape to lower temperatures. Mutagenesis studies have shown that thermotactic and chemotactic responses are mediated by the same signaling pathway, with the most abundant receptors being Tsr (responsible for warmth attraction; regulated by serine and glycine) and Tar (responsible for cold attraction; regulated by aspartate). However the mechanism for temperature-induced regulation of the receptor activity is not known (Sourijik and Wingreen 2007).

Paster and Ryu (2008) analyzed the thermal impulse response of single *E. coli* cells tethered to glass coverslips to pulses of heat, isolating the thermotactic response from the chemotactic gradients, and evaluated the CCW bias – i.e. tendency of cells to swim compared to tumble, and determined their directionality (warmth- and cold-seeking) in increasing temperature gradients. Below 31˚C, the thermal impulse was similar to the positive chemotactic impulse response (warm-seeking); above this temperature, cold-seeking behavior becomes more frequent; at 37˚C there were equal subpopulations of warm- and cold-seeking cells and at 43˚C all the cells showed cold-seeking behavior.

Based on these previous studies, this project aims to study the response of different motile organisms to temperature gradients. It attempts to develop an experimental setup for evaluating thermotaxis in the level of cell accumulation, by capillary assays, and of colony formation, by swarm plate

assays. The strains selected for this project are mainly thermophilic and moderate thermophilic isolates from seawater, brackish water, freshwater and soil environments from Woods Hole area.

2. Material and Methods

2.1. Isolation of thermophilic and moderately thermophilic strains

For practical reasons, this project required a selection of model organisms with the following characteristics:

- aerobic or facultative anaerobic;
- thermophilic or moderately thermophilic;
- fast growing in LB and M9 minimal medium;
- motile.

It is known that thermophilic bacteria are often found in cold environments, mostly as spores (e.g. Rahman *et al.* 2004; Isaksen *et al.* 1994); hence, various environments within Woods Hole area were selected as original inocula: seawater from Stony Beach, brackish water from a yellow mat pond in Gardiner Rd, freshwater from School St. Marsh and soil from Buzzards Bay Ave. 200 µL of each (soil sample was first suspended in sterile water) were inoculated on nutrient agar and R2A plates and incubated at 37, 46 and 55˚C.

After ca. 8h of incubation, the plates showed large number of colonies, except for the ones inoculated with seawater, which showed only ca. ten colonies per plate. Twenty three strains were repeatedly restreaked in rich plates (nutrient agar, R2A and LB) and maintained in the same temperature as the original plates.

2.1.1. Colony PCR and partial 16S rRNA gene sequencing of isolates

All twenty three isolates were selected for identification by partial 16S rRNA gene sequencing. Cell lysis was performed prior to colony PCR by 5 minutes of boiling in 0.05% Nonidet P40. 2 µL of the boiled sample were used as template for 16S rRNA gene amplification using the following reactants: 12.5 µL of 2x Promega master mix, 2.0 µL of 16S_8F, 2.0 µL of 16S_1492R (15 pmol each) and 6.5 µL nuclease-free water. The thermocycling program consisted of an initial denaturation step of 5 min at 95˚C, followed by 30 cycles of 30 sec at 95˚C, 30 sec at 46˚C and 1.5 min at 72˚C, and afterwards a final elongation step of 5 min at 72˚C.

Successful amplification was verified by agarose gel electrophoresis and 1.5 µL of the PCR products were purified by ExoSAP-IT clean-up reaction (0.25 µL/5 µL reaction), which consists of 30 min at 37˚C and 15 min at 80˚C. 3 µL of the ExoSAP reaction were used for sequencing.

2.2. Development of an experimental setup for microbial thermotaxis assays

Previous studies on *E. coli* thermotaxis (Maeda *et al* 1976, Salman *et al* 2006, Salman and Libchaber 2007, Paster and Ryu 2008) were based on direct microscopic observation of cells in a microchamber submitted to a temperature gradient maintained by a heating and cooling end (Figure 1a) or by a long infrared laser pulse (Figure 1b).

(a) (b)

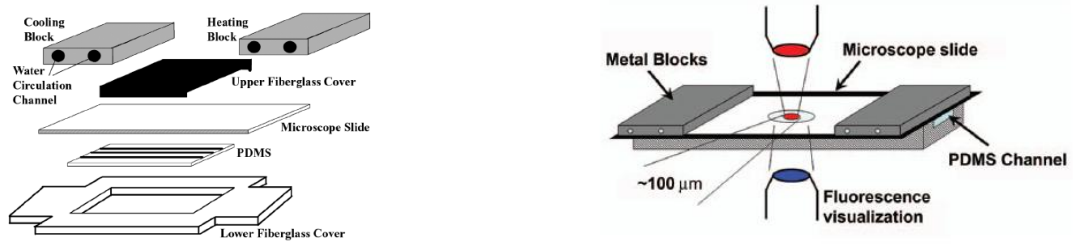


Figure 1. Examples of experimental systems for thermotaxis assays. (a) A set of narrow channels (0.07x0.002 cm), 3 cm long, microfabricated with polydimethyl-siloxane (PDMS; Salman *et al.* 2006). (b) Thin channels (700x20µm) microfabricated in PDMS and covered by 1-mm-thick glass microscope slide. An infrared laser (λ=1,480nm) is focused into the channel through the glass slide using a 32x objective (Salman and Libchaber 2007).

Time constraints for this project required the development of alternative systems. An ideal setup for a thermotaxis assay should be able to:

i. Distinguish between thermotaxis and chemotaxis (i.e. minimize nutrient gradients within a temperature gradient);
ii. Distinguish between thermotaxis and growth rate (i.e. minimize incubation time or avoid cell division)
iii. Account for orthokinesis (tendency of cells to accumulate where their speed is low);
iv. Account for differences in O_2 solubility in different temperatures;
v. Maintain a stable and steep enough gradient in a small scale.

Although challenging and unable to match all the ideal requirements at once, three systems were attempted in this project, as described below.

2.2.1. Capillary assay in an aluminum temperature gradient block

Based a similar capillary assay for chemotaxis experiments (Overmann 2005), a capillary assay for thermotaxis was envisioned as represented in Figure 2. For this assay, a microchamber was built from coverslips on a microscope slide according to Overmann (2005) and a 20cm temperature gradient surface was built from aluminum blocks (originally used as tube holders in a heating block). One end of the block was directly or indirectly heated by a heating plate, while the other end was cooled down by an aluminum block frozen at -20˚C overnight or an ice bottle at 0˚C.

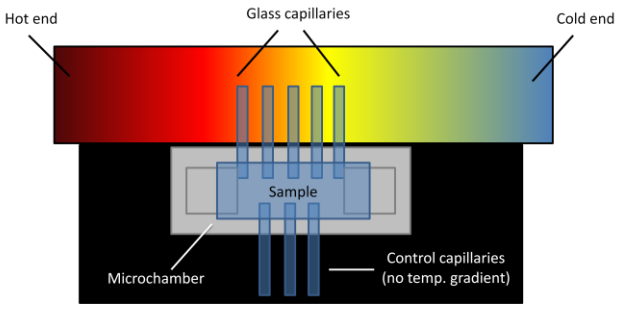


Figure 2. Idealized setup for a capillary assay in an aluminum temperature gradient block.

2.2.2. Capillary assay in a gradient thermocycler

Another capillary assay was developed using a BioRad C1000™ gradient thermocycler (Figure 3a). The machine maintains a stable temperature gradient of up to 24˚C over eight rows of wells (A-H;

$T_{min} \geq 30^{\circ}C$). Wide flat capillaries (Vitrotubes™ 0.40 x 4.0 mm, cat # 2540) were completely filled with culture grown in liquid LB, sealed with Dow Corning® high vacuum grease (to avoid drying and evaporation) and modeling clay on both ends and incubated along the thermocycler temperature gradient for 30-60min (Figure 3b). Afterwards the capillaries were examined under 10x Ph3 magnification for evidence of higher accumulation of cells in a particular position of the capillary, corresponding to a certain temperature zone in the gradient.

(a) (b)

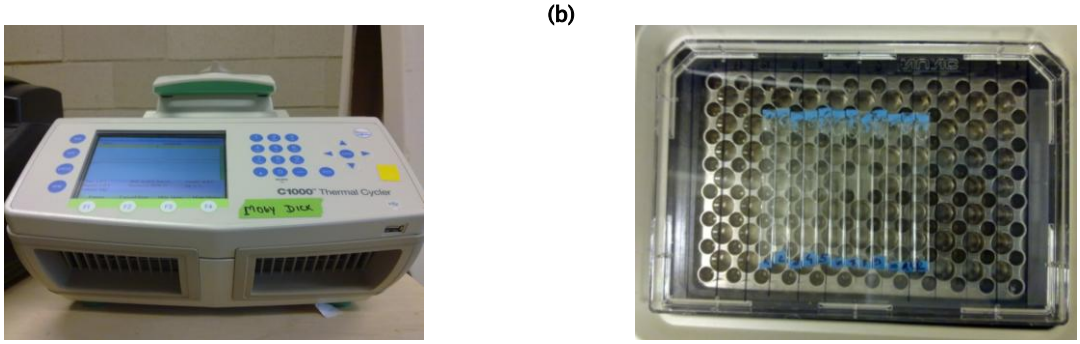


Figure 3. Experimental setup for a capillary assay in a gradient thermocycler. (a) BioRad C1000™ gradient thermocycler. (b) Capillaries incubated along the temperature gradient.

2.2.3. Swarm plate assay in a gradient thermocycler

A third thermotaxis assay setup was developed using a MJ Research PTC200 gradient thermocycler (Figure 4a), which maintains a stable temperature gradient of up to 24˚C over twelve columns of wells (1-12; $T_{min} \geq 30^{\circ}C$). A soft agar plate was strategically inoculated (Figure 4b) and incubated in the temperature gradient for up to 24h, with periodical observations of colony formation under naked eye or dissection microscope (Zeiss SteMI2000, 0.65x magnification). The soft agar swarm plates were based on M9 minimal medium (Niu *et al.* 2005 and Salman and Libchaber 2007, modified): Na_2HPO_4 42.25 mM, KH_2PO_4 22.06 mM, NaCl 8.55 mM, NH_4Cl 4.67, $MgSO_4.7H_2O$ 1.0 mM, $CaCl_2.2H_2O$ 0.1 mM, glucose 4 g/L and casamino acids 1 g/L. Most plates contained bacto agar 0.6% and Tween 80 20 µL/L, and some contained bacto agar 0.3% with no Tween 80.

(a) (b)

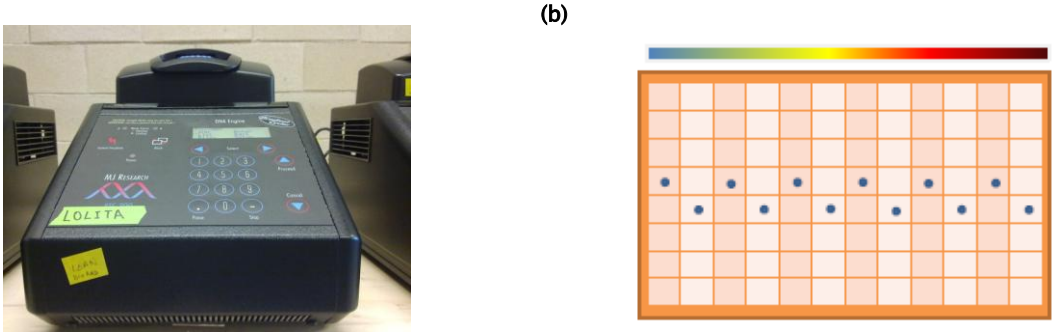


Figure 4. Experimental setup for a swarm plate assay in a gradient thermocycler. (a) MJ Research PTC200 gradient thermocycler. (b) Schematic representation of the preferred inoculation pattern: 2 µL of liquid culture are inoculated in each spot. Each column has a different temperature, and the gradient increases from left to right. Typical temperature gradients used were 30-54˚C, 36-60˚C, 46-70˚C and 56-80˚C.

3. Results and Discussion

3.1. Isolation of thermophilic and moderately thermophilic strains

Twenty three strains of thermophilic and moderately thermophilic organisms were isolated from seawater, brackish water, freshwater and soil sources from Woods Hole by incubation at 37, 46 and 55˚C on rich media. Partial 16S rRNA gene sequences were obtained for each strain. The sequences were trimmed using FinchTV, initially identified using the RDP database (http://rdp.cme.msu.edu/), aligned to the 16S rRNA database using SILVA (http://www.arb-silva.de/aligner/), and imported to ARB for further phylogenetic analyses. Figure 5 shows a maximum likelihood tree for all the isolates, indicating the phylogenetic relations which are most relevant for our purpose.

Apart of isolate Jrth04 (*Escherichia* sp.), which could be a plate contaminant, all other isolates belong to the Bacillales order within the Firmicutes. This result is not unexpected, since many Firmicutes groups are able to produce resistant spores, which may suggest that some of the thermophilic populations could be present in these colder environments in a dormant state, i.e. not active *in situ*. Cryptic growth is another hypothesis for the presence of thermophiles in environments with temperature below the range experimentally determined.

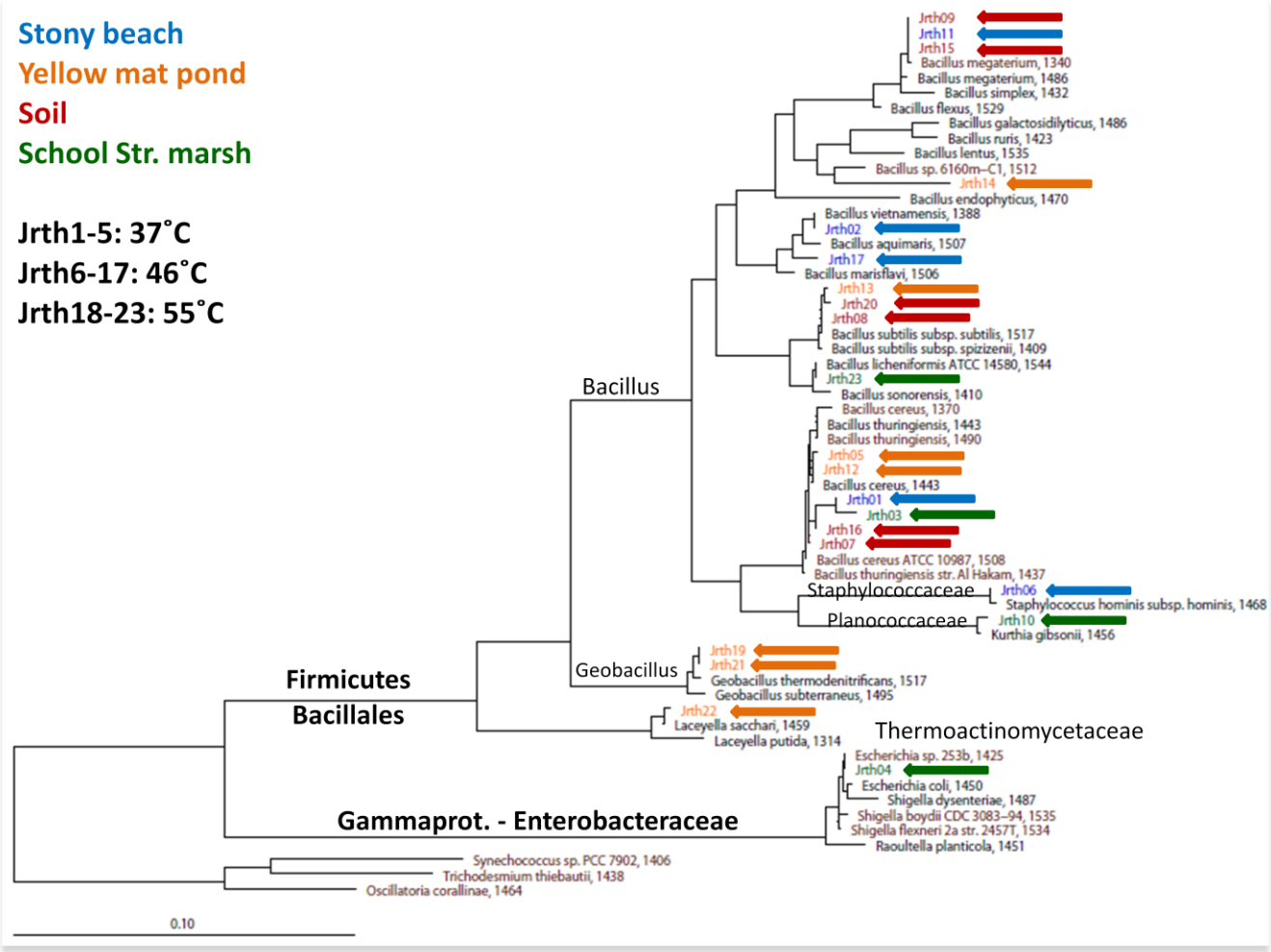


Figure 5. Maximum likelihood tree (DNA PhymL) for the thermophilic and moderately thermophilic isolates. The original inoculum source and the incubation temperatures for each strain are indicated by the colors and numbers, respectively.

Microscopic observation of all isolates was performed to characterize cell morphology and determine which strains were motile. Figure 6 shows the main morphologies. Most strains consist of short or long rods; only Jrth06 (*Staphylococcus hominis*) and Jrth22 (*Laceyella sacchari*) show particularly distinct morphology and were clearly non-motile.

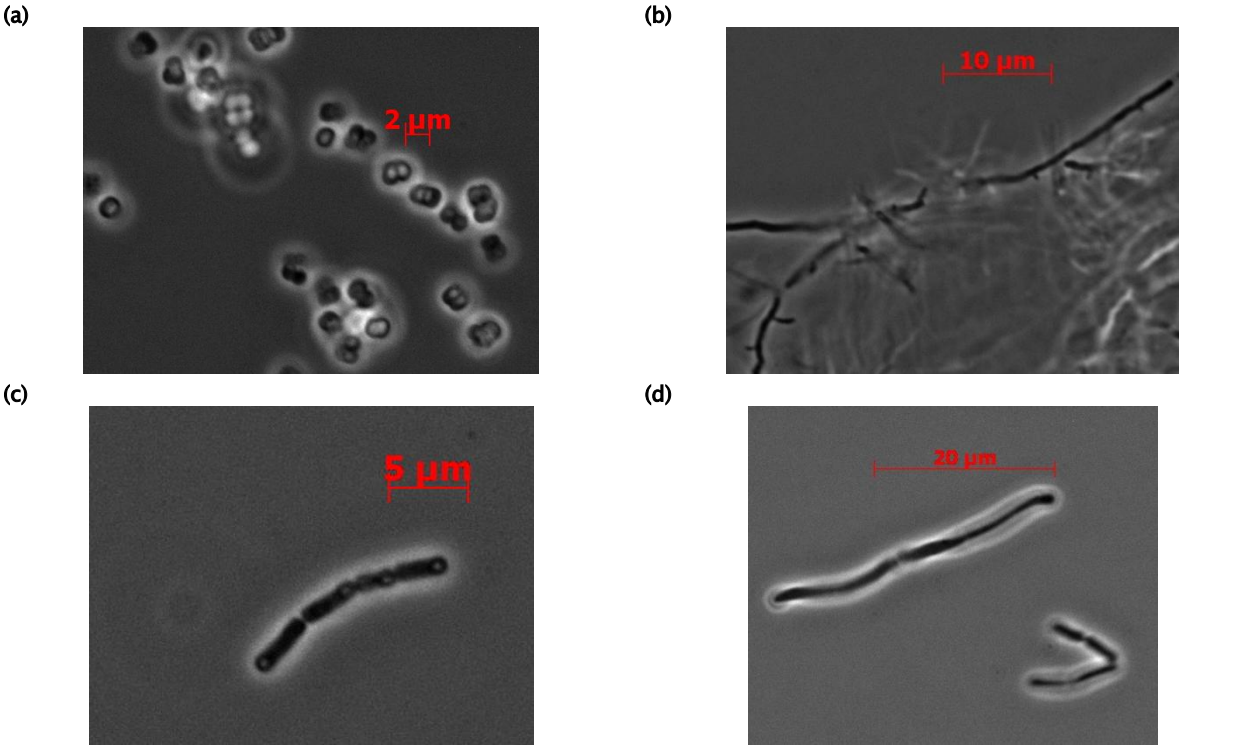


Figure 6. Examples of cell morphologies. (a) Jrth06 (*Staphylococcus hominis*), (b) Jrth22 (*Laceyella sacchari*), (c) Jrth12 (*Bacillus cereus*), (d) Jrth15 (*Bacillus megaterium*).

Five isolates were selected for swarm plate assays and tested for their growth temperature range by incubating in the same conditions (same inoculation strategy and M9 0.3% agar) as swarm plates incubated in the thermocycler, but at the constant temperatures, as shown on Table 1.

Table 1. Non-gradient swarm plates of selected isolates and *E. coli*. RT = room temperature, ca. 24˚C.

Strain	RT	37˚C	46˚C	55˚C
Shigella sp.	++	+++	+++	-
E. coli	++	++++	++++	-
B. megaterium	++	++	+++	-
B. cereus	++++	++++	++++	-
B. licheniformis	+	+++	++++	++++
G. thermodenitrificans	-	-	-	++

3.2. Development of an experimental setup for microbial thermotaxis assays

3.2.1. Capillary assay in an aluminum temperature gradient block

Despite the dedicated effort, it was not possible to achieve a stable temperature gradient in an aluminum gradient block (Figure 7). The main problem was to cool one end with the same efficiency as heating the other end. The heating plate used was highly efficient, so one partial solution was to use a hot water bottle to indirectly heat the aluminum surface or a small flat aluminum piece. For the cold end, a frozen aluminum block was very efficient in removing heat, but only for a few minutes, as it clearly warms up to room temperature very fast. An ice bottle was another partial solution, since it can be easily maintained at 0˚C and substituted when all ice melts. Gradients of ca. 25-30 or 33-43˚C were achieved with relative stability, however it was not enough for a proper thermotaxis assay. Improvements would be certainly necessary in the future.

Figure 7. Process of development of a small aluminum temperature gradient block for a microchamber assay.

3.2.2. Capillary assay in a gradient thermocycler

This experimental setup showed more promising results. The major advantage of a capillary assay is that it minimizes the effect of different growth rates in different temperatures, since the incubation time could be as short as 30-60 min, and the visualization by 10x Ph3 microscopy is rather convenient. The combination with a gradient thermocycler makes it more promising as it maintains a very stable and adequately steep temperature gradient, which can be easily regulated.

A drawback of capillary assays in general is the production of an O_2 gradient, which may influence the results, as reported by Maeda *et al.* (1976) with a similar system, or the inability to test strictly aerobic strains, which may die or not move under anoxic conditions. As for the thermocycler, one drawback is that the minimum gradient temperature must be at least 30˚C, which limited the selection of strains for this assay and motivated the isolation of thermophiles and moderate thermophiles. A second intrinsic drawback is that the temperature in the thermocycler wells (which is what can be regulated) is most likely

not the same as in the surface, i.e. the indicated temperature is not the real temperature experienced by the cells. The temperature of the lid certainly has an influence as well. Since it was not possible to accurately measure the real temperature and the set temperature (i.e. temperature in the thermocycler wells) is the only close reference to it, the temperature of inoculation spots or columns will be mentioned in the text between quotation marks.

Another drawback for this specific setup is that it was not possible to keep capillaries on the temperature gradient connected to a microchamber outside of the gradient, i.e. one capillary per temperature, which would favor the evaluation of accumulation of cells. Therefore, the strategy was to create a temperature gradient within a single capillary. The wide capillaries (Vitrotubes 0.40 x 4.0 mm) were chosen in order to minimize the effect of nutrient depletion.

It was not possible to visualize any distinguished accumulation of cells in the preliminary tests with *E. coli*, Jrth02 (*B. vietnamensis*), Jrth04 (*Escherichia* sp.), Jrth10 (*Bacillus* sp.), Jrth11 (*B. megaterium*), Jrth12 (*B. cereus*) and Jrth23 (*B. licheniformis*). However, this negative result is most probably due to very high initial density of cells in the capillary. Therefore, this setup seems promising and also requires improvements. The first step in future work would be the optimization of the initial cell density for each strain, as well as the optimization of incubation time.

3.2.3. Swarm plate assay in a gradient thermocycler

This experimental setup provided various interesting results (Figure 8). Once again, the thermocycler presents the same advantages (stable and steep temperature gradient) and disadvantages (minimum gradient temperature is 30˚C, difficult to measure the real temperature on the sample). In comparison to capillaries, swarm plates present the disadvantage that they are influenced by differential growth rate, which could mask thermotaxis. On the other hand, they are not influenced by O_2 depletion, and the analysis of the plates lead to several other interesting observations, such as temperature preference, growth rate and colony formation patterns at different temperatures, as reported below.

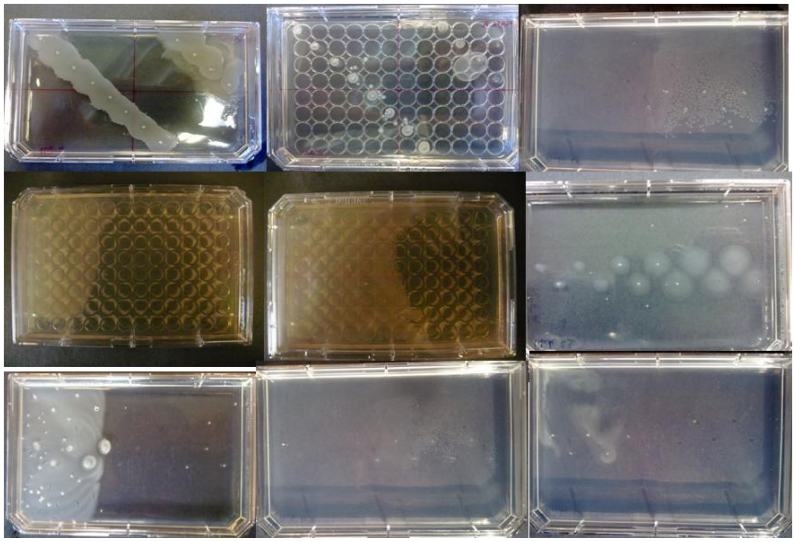


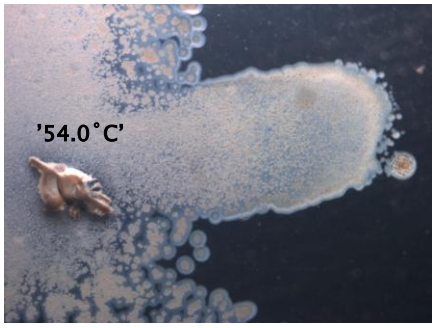
Figure 8. Examples of swarm plate assays in a gradient thermocycler.

Indications of thermotaxis in B. licheniformis

The isolated strain of *Bacillus licheniformis* (Jrth23) repeatedly showed growth patterns in swarm plates that may indicate thermotaxis ability (Figure 9). After 20h of incubation, plate 1showed lawn growth, with no isolated colonies, from the cold end at '36˚C' until ca. '54˚C'. However, the inoculation spot at '54.0' generated a protuberant accumulation of cells towards higher temperature, which reached further than the limits of the lawn growth (Figure 9a). Additionally, the next inoculation spot, at '57.3˚C', showed isolated colonies towards higher temperature as well (Figure 9b). The presence of isolated colonies away from the inoculation spot suggest that cells swim away from their source, settle at certain points and generates a colony. The 'decision' of settling down could be related to both chemo and thermotactic responses.

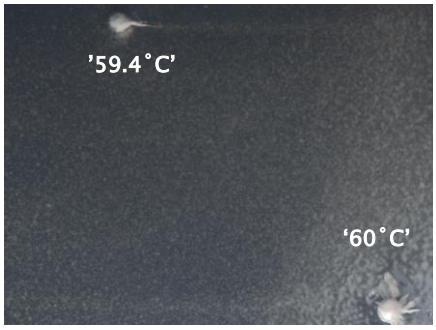
The second and third plates of *B. licheniformis* also showed growth patterns that may indicate thermotaxis. After only 6h of incubation, begin of lawn growth could be already observed, but in different densities for each temperature zones. Figure 9c and 9d show not very dense growth around the last two spots on the plate, '59.4' and '60˚C', except for a distinguished area on the right of the very last inoculation spot, with dense accumulation of cells. It is possible that the dense growth is caused by cells that moved from the inoculation spot towards the edge of the plate (which can be seen on the right side of Figure 9b). Since this is the last inoculation spot, going to the right suggests escaping from the high temperature directly below the inoculation spot (thermocycler column 12), towards a slightly lower temperature, as the edges are cooler. Plate 3, a duplicate of plate 2, also shows a distinct denser path formed from the inoculation spot towards the edge of the plate, even though there is some growth distributed circularly around the inoculation spot (Figure 9e and 9f).

(a)



(b)

(c)



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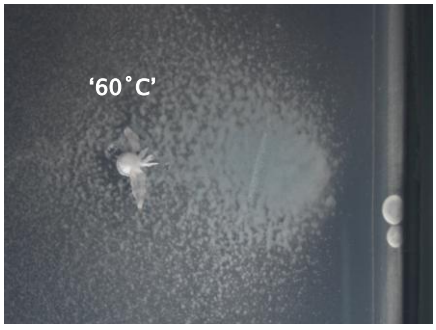
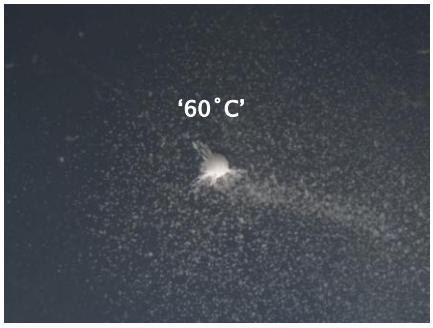


(e)



(f)

Figure 9. Indications of thermotaxis in swarm plates of *Bacillus licheniformis.* (a)–(b) Plate 1, '36–60˚C', 20h of incubation. (c)–(d) Plate 2, '36–60˚C', 6h of incubation; (d)–(e) Plate 3, '36–60˚C', 6h of incubation. The temperature gradient increases from left to right. The temperature value indicated in quotation marks is the temperature in the well directly below the inoculation spot. See text for further details.

These patterns should not be taken as clear evidence of thermotaxis in *B. licheniformis*; more experiments and controls must be done to assure that the distinguished accumulation of cells can be pinpointed to thermotaxis. Nevertheless, it is an interesting indication that motivates further work.

Calculating growth rates from colony area in a temperature gradient

Besides some indications of thermotaxis, the swarm plate assays also provided interesting observations on growth and colony formation patterns. For instance, the increase in colony area of *Bacillus thermodenitrificans* (Jrth21) on M9 medium with 0.3% agar (Figure 10) can be used for estimating growth rate simultaneously at all twelve temperatures.

For this analysis, two swarm plates of *B. thermodenitrificans* were incubated at the same time on a '36–60˚C' and a '46–70˚C' gradient. Pictures of each of the twelve colonies were taken after 4.5, 6 and 9h of incubation with a dissection microscope at 0.65x magnification. The radius of the colony, which is fairly circular, was used to calculate the area, assumed to be proportional to the cell number.

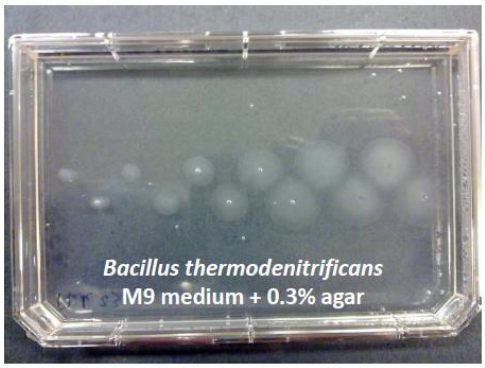


Figure 10. *B. thermodenitrificans* swarm plate, gradient between '46–70˚C' (left to right) after ca. 20h of incubation.

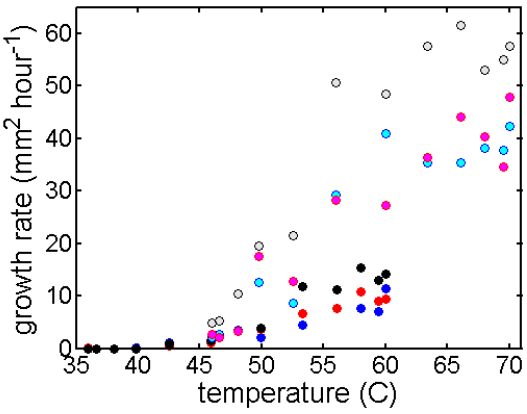


Figure 11. Growth rate per distinct temperature in two *B. thermodenitrificans* swarm plates: Blue/light blue: 4.5h of incubation; red/pink: 6h of incubation; black/white dots: 9h of incubation. Blue, red and black: plate from '36-60˚C' gradient; light blue, pink, white: plate from '46-70˚C'. The indicated temperature is the temperature on the thermocycler wells.

Figure 11 shows the growth rate for of each colony according to the temperature gradient on both swarm plates. The data indicates that the growth rate tends to increase in the first 9h of incubation, and the growth rate is higher at higher temperatures, as expected. It also shows that between '46' and '60˚C', where the gradient of both plates overlap, there is almost no overlap of the data points. This might be due to the fact that the indicated temperature (well temperature) is not the real temperature on the plates, as discussed earlier. Moreover, the wells on the central part of the plate might be less influenced by fresh air from the sides, and the lid temperature is different for each gradient set: it is 53˚C for a '36-60˚C' gradient and 63˚C for a '46-70˚C' gradient. The lid temperature influences the temperature of the air above the plate, and therefore the temperature on the surface.

Although there was no overlap between the shared temperature window, this analysis is valid for evaluating the real temperature on the plates and stimulating the use of swarm plates for growth rate studies simultaneously at twelve distinct temperatures.

4. Conclusion

The work here presented shows that:

- *Bacillus licheniformis* swarm plate assays show indications of thermotaxis.
- The experimental setup for the proposed thermotaxis assays must be largely optimized in order to confirm such observations and evaluate other organisms.
- Viable thermophiles and moderate thermophiles are present in seawater, brackish water, freshwater and soil environments in Woods Hole.
- Swarm plate incubations in a gradient thermocycler can be an interesting technique for evaluating colony development in different simultaneous temperatures.

5. Acknowledgments

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